Investigations into the Role of cGMP in Mediating the Effects of Extracellular Nucleotides on Root Hair Growth in *Arabidopsis thaliana*

By

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Abstract

Investigations into the Role of cGMP in Mediating the Effects of Extracellular Nucleotides on Root Hair Growth in *Arabidopsis thaliana*

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The eATP pathway begins a cascade of events which includes the involvement of nitric oxide synthase (NOS) and nitrate reductase (NR) in the production of nitric oxide (NO). Research has shown that SNAP (*S*-nitroso-*N*-acetylpenicillamine) and NONOates (diazeniumdiolates) promote the availability of NO and, with the addition of guanylate cyclase, form cyclic guanine monophosphate (cGMP), and root hair growth is promoted. Phosphodiesterases (PDE) break down the cGMP and agents such as IBMX and Viagra inhibit the PDEs thereby inhibiting root hair growth. Several questions remain to be answered. How much cGMP is necessary for the promotion of root hair growth? Is there an optimal concentration of cGMP which stimulates root hair growth, above which is inhibitory, or below which is ineffective? Is there a "non-hydrolyzable analog" of cGMP which is more effective at promoting root hair growth? Is it possible to see inhibition of root hair growth with exposure to a known inhibitor, such as ATPγS, and then reverse the inhibition with a "non-hydrolyzable analog" of cGMP? Answering these

questions is the substance of this research and the answers will provide direction and understanding to the growth-promoting and regulatory role eATP plays in signal transduction pathways in plants. With the hypothesis asking whether the effects of NO on root hair growth is cGMP-dependent or cGMP-independent we found that there is no consistent concentration of non-hydrolyzable cGMP analog which promotes root hair growth. Additionally we found that the 8-Br-cGMP analog promotes root hair growth more consistently in *Arabidopsis thaliana* than its counterpart, dibutyryl cGMP. We substantiated previously published results showing an inhibition of root hair growth when root hairs were exposed to high concentrations of ATPγS. Based on these results we believe the promotion of root hair growth in *Arabidopsis thaliana* to be mediated independently of cGMP.

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Chapter 1: *Introduction*

Communication is an obvious necessity in a complex multicellular system. Because plants and animals are multicellular, communication systems are extremely important. Communications between organisms are important for the survival of the species. Equally important are the communications between the cells of the organism. Plants and animals are obviously very different organisms and each responds to its environment in very different ways. Plants, being stationary organisms, must respond to every environmental change that occurs where they are rooted. Animals, on the other hand, can move toward the positive and away from the negative environmental stimuli. However, just because plants and animals respond differently to their macroenvironments, they may respond similarly to their microenvironments. Signaling systems known to occur in animals are now being explored in plants.

Intercellular communications include the same requirements as traditional communication systems: a sender, a receiver, and a signal. Intercellular systems use three additional systems, all of which are chemically based: signal reception, signal transduction, and cellular response. Once a signal is received by a cell, the signal is transduced, converting the signal to a form understood by the cell and allowing the cell to make a specific response. In the case of plant intercellular communications, the sender is any stimulus a plant may encounter in the environment in which it is growing. These stimuli come in many forms, from all parts of the environment, and are grouped into external and internal stimuli. Cellular communications from outside the plant organism include abiotic factors such as drought, salinity, extreme temperature changes and biotic

factors such as insect predation and injury, while those occurring within the plant organism include factors such as mechanical stress and plant growth and development hormones.

Each stimulus produces a physiological response through a cascading pathway of chemical reactions. When the plant receives the stimulus, a conformational change occurs in the receptor protein which often resides in the plasma membrane, causing the receptor protein to become active by changing the receptor's shape. Only specific receptor proteins react and change shape when exposed to the stimulus, even though there are many other potential receptor proteins in the cell. Once the protein receptor changes shape it then interacts with other cellular components.

The cell cytoplasm is an aqueous environment and the cell membrane is a hydrophobic environment, and some receptor proteins and signal molecules are water-soluble and some are more lipid-soluble. Signaling pathways can begin in the cytoplasm or in a membrane. As an example of a signaling event that begins in the cytoplasm, when a light signal activates the phytochrome receptor in a plant cell, the photoactivated protein both initiates an increase in the cytoplasmic calcium ion concentration ($[Ca^{+2}]$) and then moves to the nucleus where it induces the phosphorylation of transcription factors. These processes lead to a change in the expression of genes responsible for the de-etiolation, or greening, response in the plant. From this example, the reader can understand that multiple and complex pathways are usually involved in any signal transduction method of communication. These mechanisms include the previously mentioned calcium-based signaling, which involves ion-channel transporters, G-protein-

mediated-signaling, which uses G-protein-linked receptors, phospholipase C-mediated signaling, using inositol phospholipids, and receptor kinases, which activate multiple different transduction pathways via protein kinases that transfer a phosphate group from ATP, the energy molecule of the cell, to a serine, threonine, histidine or tyrosine amino acid on a protein substrate. With so many different signaling pathways, mechanisms, and at least sixteen classes of receptors with signal transduction mechanisms (Pollard and Earnshaw, 2008), one can readily understand the need to focus on one pathway and its inherent complexities in one organism to begin grasping how that mechanism may work in other organisms.

Intracellular ATP is well known as the energy molecule in the cell. However, ATP also functions as a signaling agent outside the cell and the regulatory role extracellular ATP (eATP) plays is less well understood. For more than twenty years, scientists have studied the effects of eATP in animal systems and, according to Roux and Steinebrunner (2007), "over 470 articles have been published on the topic in 2006 alone." So what is the role of eATP as a regulator in the plant cell? What does eATP regulate and how? Although ATP is made in the mitochondria and chloroplasts inside the plasma membrane borders of plant cells, there are several mechanisms whereby it can leave the cytoplasm and regulate cellular responses. One obvious way would be that ATP escapes into the extracellular matrix (ECM or wall) from a break in the plant cell tissue. Once outside the cell, eATP induces diverse physiological changes.

Lew and Dearnaley (2000) found that the nucleotides, ATP and ADP, depolarized the plasma membrane of *Arabidopsis thaliana* root hairs. They also learned that

phosphates had no effect on membrane potentials, indicating that the nucleotide effects were not due to the hydrolysis and uptake of phosphates. Several researchers have shown that applied ATP and its poorly hydrolysable analogs increase the cytosolic Ca^{+2} concentration ($[Ca^{+2}]$) in plant tissues. Additionally these researchers have shown that these effects are not due to a lowered pH, the products of hydrolysis of the applied nucleotides, or the chelation of divalent cations (such as Ca^{+2}) in the ECM. Because the earliest step in animal signaling systems is an increase in $[Ca^{+2}]$ resulting from the activation of either ion channel-linked or G-protein linked purinoceptors, researchers have surmised a similar beginning to the eATP signaling pathway in plants. Purinoceptor-like genes have been found in algae; however researchers are continuing to search for the genes encoding proteins with receptor activity for eATP in higher plants.

Downstream from the initial eATP stimulus causing the increase in $[Ca^{+2}]$, researchers have focused on (known) "calcium-regulated NADPH oxidase activity and found that as little as 250 nM ATPγS could induce an NADPH oxidase-dependent increased production of superoxide (O_2^-), a recognized mediator of wound responses in plants." (Roux and Steinebrunner, 2007) Continuing research has proven that plants react similarly to the various known inhibitors of ATP signaling in animal systems, suggesting that the responses to $[Ca^{+2}]$ are initiated by similar purinoceptors. Specific to this research is the "eATP-induced increase in the cytosolic Ca^{+2} concentration (which) activate(s) the calmodulin-regulated nitric oxide synthase resulting in nitric oxide production, which is a known downstream mediator of ATP signaling in animal cells" (Roux and Steinebrunner, 2007).

Animal systems also use hydrolytic enzymes with active sites on the external surface of the plasma membrane, called ectonucleotidases, to hydrolyze eATP, thereby reducing the eATP concentration. Upon altering the expression of two apyrases found in *Arabidopsis thaliana*, Wu et al. (2007) found that these two ectonucleotidases play a key role in controlling growth in *Arabidopsis*. Steinebrunner et al. (2003) found that inhibition of pollen germination resulted from suppressing the apyrase levels via a double knockout of the two enzymes, AtAPY1 and AtAPY2. Continued research in several groups suggests there is an "optimal concentration of eATP for growth" (Roux and Steinebrunner, 2007) and indicates that concentrations above and below this will inhibit growth.

Following this train of thought and progressing farther downstream from the stimulus which initiates the eATP response pathway, one finds many explorations into the role of the $[Ca^{+2}]$/Calmodulin influence on the production of nitric oxide (NO). Notably, Foresi et al. (2007) have demonstrated a direct link between eATP concentrations ([eATP]) and the production of NO in tomato cell suspensions using a fluorescence technique which detected the presence of NO following cellular exposure to ATP in various concentrations. There is a weak fluorescence in the absence of applied ATP concentrations, suggesting a basal low level of NO production in unstimulated cells. In the presence of NO scavengers and NO synthase (NOS) and nitrate reductase (NR) inhibitors, NO production was diminished in response to the ATP dosage applied. These results of Foresi et al. (2007) strongly link the exogenous ATP applications to NO production and demonstrate that, as in animal systems, NOS activity occurs in plant

systems, as well as the presence of NO produced by NR and both enzymes play roles in the eATP control of growth and development in plants.

Wu and Wu (2008) also found a direct link between the exogenous application of ATP and its non-hydrolyzable analog, ATPγS, in the production of NO, but they further investigated the role of Ca^{+2} and H_2O_2 as signaling agents in the eATP signaling pathway. Not only did they find an exogenous ATP dose-dependency with an optimum range of [ATP] near 100μM, which was mimicked with ATPγS, but also they found that "ATP-induced NO production was blocked by Ca^{+2} antagonists (and) not affected by a protein kinase inhibitor." Additionally, they found that the ATP-induced rapid increase in Ca^{+2} levels, while dependent on NO, were not dependent on H_2O_2 suggesting an NO-implicated role in "ATP-induced responses" and a link between ATP signaling and Ca^{+2} and reactive oxygen species (ROS) in plant communication systems.

Upon learning that increasing the extracellular concentrations of ATPγS inhibited pollen germination and pollen tube elongation and knowing that while growing, pollen tubes are sensitive to NO, Reichler et al. (2009) investigated the two pathways, eATP signaling and NO signaling, and their influence on each other in pollen germination and pollen tube elongation. Their results showed that exogenously applied ATPγS induced an increase in cellular NO, the chemical agonists of the NO signaling pathway (SNAP and NONOate) lower the extracellular ATPγS ([eATPγS]) threshold inhibiting pollen germination, ODQ, an antagonist of guanylate cyclase, blocks the inhibiting effects of ATPγS on both pollen tube growth and pollen germination, and, in *nia1nia2* mutants possessing diminished NO production, the effects of exogenously applied ATPγS were

blocked. Their results show that the suppression of the pollen germination and pollen tube growth is mediated by the NO signaling pathway.

When a plant experiences a wounding stimulus, several pathways are initiated. Rather than chasing rabbits and explaining all the potential plant responses, only the eATP signaling pathway is described. The wound is the stimulus which initiates the break in the plant's cell walls causing the release of cytoplasmic ATP into the extracellular matrix. However, the question of how eATP accumulates in ECM during growth and development was answered by Kim et al. (2006) who showed that actively growing root hairs exhibit increasing ATP concentration in their ECM as they grow. Plant growth and development hormones include auxin, or indoleacetic acid (IAA), found at apical meristems and in seed embryos promoting and regulating root growth, stem elongation, apical dominance, and photo- and gravitropisms; cytokinins, a group of hormones involved in actively growing plant tissues such as roots, embryos, and fruits; gibberellins, a group of hormones involved in stimulating the growth and development of leaves and stems; and brassinosteroids, chemically similar to those found in animals and responsible for cellular elongation and division in stems and seedlings at extremely low concentrations (10^{-12} M). Therefore, the eATP is not acting solo in its role as a growth regulator. The almost microscopic *Arabidopsis thaliana s*eeds planted on a Murashige-Skoog agar medium and germinated in a growth chamber have enough plant growth hormones to produce roots and shoots capable of being studied. Because Kim et al. (2006) so eloquently demonstrated the existence of eATP in the ECM of actively growing root hairs, many questions were raised as to how the eATP gets into the ECM,

what does the eATP do, how does eATP progress through to stimulation of plant growth and development.

The eATP pathway begins a cascade of events which includes the involvement of NOS and NR in the production of NO. Research has shown that SNAP (*S*-nitroso-*N*-acetylpenicillamine) and NONOates (diazeniumdiolates) promote the availability of NO and, with the addition of guanylate cyclase, the enzyme that works on guanine triphosphate (GTP) breaking it down by removing two phosphates and forming cyclic guanine monophosphate (cGMP), the cycle continues and root hair growth is surmised. Phosphodiesterases (PDE) break down the cGMP and agents such as IBMX and Viagra inhibit the PDEs thereby inhibiting root hair growth. Several questions remain to be answered. How much cGMP is necessary for the promotion of root hair growth? Is there an optimal concentration of cGMP which stimulates root hair growth, above which is inhibitory, or below which is ineffective? Is there a "non-hydrolyzable analog" of cGMP which is more effective at promoting root hair growth? Is it possible to see inhibition of root hair growth with exposure to a known inhibitor, such as ATPγS, and then reverse the inhibition with a "non-hydrolyzable analog" of cGMP? Answering these questions is the substance of this research and the answers will provide direction and understanding to the growth-promoting and regulatory role eATP plays in signal transduction pathways in plants.

Chapter 2: Literature Review

The article authored by Clark et al. (2001) provides an excellent starting point for a researcher interested in exploring the various signal transduction mechanisms in plants. The article reviews previously published literature covering the mechanisms of calcium-based signaling, including discussions of the measuring and regulation of Ca^{+2} in cells, the targets of Ca^{+2} signals and Ca^{+2}-activated proteins, CaM (calmodulin), annexins, and protein kinase C. Additionally, the authors continue their discussion of plant signaling mechanisms with a discussion of the mechanisms involving inositol phospholipids and G protein-mediated signaling. All the mechanisms reviewed are very complex, working in multi-layered networks and never in isolation from other signaling pathways, and affect all aspects of plant growth and development. The authors do include a discussion of recently published findings on three extracellular agents in these signaling pathways: extracellular CaM, extracellular ATP, and integrin-like receptors. The article summarizes well the research published previous to January 2001.

More than half a decade later new knowledge has been gained, but this field continues to be wide open in terms of understanding the complex signaling pathways in plants, as indicated in a more recent review by Roux and Steinebrunner (2007). This article is another one of the first I read authored by my research supervisor. A very concise and easily understood primer describing the roles of extracellular ATP (eATP) as a signaling molecule in plants, the article raises questions, reviews potential answers published by various researchers, reviews literature describing similarities between eATP

signaling and responses seen in animals and plants, and provides a direction for future research.

Demidchik et al. (2003) were among the first to investigate possible eATP-induced signaling pathways. These authors hypothesized that, because Ca^{+2} is a downstream second messenger from eATP in animal cells, the eATP could alter cytosolic calcium ion concentrations in the cells of excised roots of *Arabidopsis thaliana* plants. Experimental procedures for measuring the $[Ca^{+2}]_{cyt}$ were described in a previously published paper, but Figure 1 showed the effect of various externally applied purines, UTP, and nucleotide analogs on the $[Ca^{+2}]_{cyt}$ of *Arabidopsis* root cells, just as it does in animal cells. A few months later Jeter et al. (2004) showed that eATP could also induce increased $[Ca^{+2}]_{cyt}$ in aerial portions of intact seedlings, and that these changes were linked to increased expression of genes typically turned on by stress stimuli.

Regarding the question of how ATP moves into the ECM, the chapter by Roux et al. (2001) was very informative. The authors provide three exit strategies for eATP's presence in the ECM. First, eATP leaves the plant cells when the cells undergo autolysis, or self-destruction. Second, eATP leaves the plant cells when the cell's vesicles merge into the plasma membrane and the vesicle contents are then dumped into the ECM. Lastly, the eATP leaves the cell through specialized transport proteins, such as the ABC transporters which mediate either directly or indirectly the intracellular adenylate accumulations involved in signaling mechanisms. Evidence for these ATP exit strategies was subsequently provided in research publications by Song et al. (2006), Kim et al. (2006) and Wu et al. (2007).

Song et al. (2006) found that cytoplasmic ATP is released into the ECM at wound sites in leaves of *Arabidopsis,* and can mimic the wound stimulus by inducing the production of reactive oxygen species (ROS) in a dose-dependent manner through the mediation of NADPH oxidase. Kim et al. (2006) followed up the Song et al. findings by showing that ATP is also released at sites of rapid growth. They set the world of eATP signaling in plants on fire with their remarkable research which provides "definitive experimental evidence for (the) presence or (the) explanation as to how…a polar molecule (eATP) could exit the plant cell and what physiological role it may play in plant growth and development." Relating the well-documented evidence of eATP in animal systems, the authors devised a novel reporter linking the "ATP-requiring enzyme luciferase" to a cellulose-binding domain peptide and used the reporter to allow "visualization of eATP in the presence of the substrate luciferin." Luciferase activity was noted in the extracellular matrix (ECM) between epidermal cells in actively growing roots of *Medicago trunculata* plants. Pre-treating the seedlings in water for one hour with several pharmacological agents such as a nonhydrolyzable ATP, potato apyrase, $GdCl_3$, BAPTA, $LaCl_3$, and brefeldin A allowed the authors to demonstrate that ATP is prevalent at root tips and actively growing areas of the plant, that the CBD-Luciferase protein complex is distributed evenly at root hair surfaces and is associated strongly with the cell wall, that ATP secretion is calcium dependent, that ATP plays a role in the reactive oxygen species (ROS) in root hair tips, and that this enhanced eATP secretion is found in actively growing epidermal and cortical cells of etiolated hypocotyls and in the zone of elongation in root apices yet is minimal in the more mature areas of roots. This

landmark study proves unequivocally that eATP is a central player in plant cell signal transduction pathways.

A key paper that more directly showed the relationship of eATP and ectoapyrases to growth was that of Wu at al. (2007). These authors report the highest expression of apyrases in actively growing tissues in *Arabidopsis.* Two genes with a high degree of similarity between them, *APY1* and *APY2*, were analyzed in this study using β – glucuronidase during seedling development under various light treatments. The authors state "(t)hese results imply that APY1 and APY2, like their homologs in animals, act to reduce the concentration of extracellular nucleotides, and that this function is important for the regulation of growth in Arabidopsis." Further evidence for the role of eATP and ectoapyrases in growth control came from the report of Wolf et al. (2007). These researchers found double knockout pollen could not germinate, but they could restore pollen germination in the "apyrase T-DNA double knockouts (DKO) *apy1-1/apy1-1; apy2-1/apy2-1* by complementation with *AtAPY2* under the control of a pollen-specific promoter." The DKO plants produced had many developmental defects including the lack of functioning root and shoot meristems and unlobed pavement cells and stomatal clusters. The addition of a dexamethasone-(DEX)-inducible promoter produced less severe types of developmental defects in plant cells, but these mutants were also seedling lethal. These authors demonstrated the importance of the *AtAPY 1* and *AtAPY2* genes in normal plant development. Using reverse genetic and biochemical approaches, Riewe et al. (2008) investigated the role of potato-specific apyrase. Silencing the apyrase gene family resulted in many phenotypic changes in the transgenic lines to include "a general

retardation in growth, an increase in tuber number per plant, and differences in tuber morphology." These authors demonstrated the role of the enzyme in the apoplast, and provide evidence for its direct involvement in regulating growth and development in plants.

The publications showing that eATP could regulate growth raised the question of how? What signaling steps connected eATP to growth changes? My research focused on the role of NO in mediating the eATP effects on growth via cyclic GMP (cGMP). In preparation for this research, I found background information regarding the activity of NO, its formation, and the enzymes that work on the eATP substrate to form the NO. Stöhr et al (2001) found "nitrite-reducing enzyme activity that resulted in nitric oxide (NO) formation" in the purified plasma membranes of tobacco roots. This enzyme activity was not found in the plasma membrane vesicles and protein fractions of leaves. This paper reveals another possible explanation for the presence of NO in plant roots. This group also separated the two enzymes in question, PM-NR (plasma membrane nitrate reductase) and NI-NOR (nitrite: NO-reductase) with a western blot analysis.

Continuing to search for answers to the role of NO in plant signaling systems led me to Yamasaki (2005). This paper compares the two nitric oxide (NO) production pathways in plants: the arginine pathway (the more complex path where oxygen and enzymes are required) and the nitrite pathway (the simpler path where chemicals reduce the nitrite to NO). The author also proposes a new hypothesis, the ONS hypothesis, which seeks to explain the simple, yet complex interactions between reactive nitrogen species (RNS), reactive oxygen species (ROS), and reactive sulphur species (RSS) in

plants. Because plants are seen by the author as "open systems" having closer contact with the nitrogen, oxygen, and sulphur free radicals existing in the atmospheric environment than do animals, the author proposes a more integrative research process when investigating the NO signaling pathways in plants, including comprehensive studies beginning with molecular interactions to those interactions between organisms. Identifying the underlying mechanisms relating the three reactive series would provide a foundation to understanding the basic "balance science for three distinct dynamic elements (ROS, RNS, RSS)." ROS are generated in the chloroplasts as a toxic byproduct of photosynthesis and CO_2 assimilation; RNS are generated as a byproduct of nitrate assimilation; RSS are a proposed byproduct of sulphur assimilation making all three mutually interactive and causing complexities in the study of free radical biology. Previous thinking and study concluded that NO is a free radical and a toxic environmental pollutant. However further and more recent study has suggested NO as an "essential molecule endogenously produced in the cells…act(ing) as a plant hormone equivalent to ethylene; that is, as a gaseous signal transmitter." The proposed ONS hypothesis should aid in the further exploration of the complexities of NO signaling pathways and the accompanying organismal-environmental interactions.

In the progress report by Lamotte et al. (2005) a great schematic representation of the NO signalling pathways in plant cells shows all the (known) routes for NO synthesis. The authors also provide a short description of several questions that needed to be answered at the time of their report to include a study of the chemistry involved in the reaction catalysed by NOS, understanding the mechanisms of the NO signaling pathways

by studying the protein, AtNOS1, its location in plant cells, and other proteins with which it may be involved, a study of the *AtNOS1* gene, its regulation and expression in the growth and development of plants and its role in the environmental responses of plants. Lastly the authors think a study into the identification of the NO targets in plants would be beneficial.

Foresi et al. (2007), in their scientific correspondence, show the results of several experiments in the study of the effect of exogenous ATP on nitric oxide (NO) production in tomato cell suspensions. Using fluorescence and a microwell fluorometer over a 2-hr. time period, this group demonstrates that 1mM concentrations of ATP generated high levels of NO as expressed in arbitrary units (AU) of green fluorescence. The group continued their study by using an ATP concentration gradient and demonstrated an increase in NO generation (as expressed with fluorescence) with an increase in ATP concentration. Also remarkable is the fact that the cell suspensions with no ATP exposure incurred a slight increase in NO generation, thus demonstrating a cellular source of minimal NO production. To insure that a cellular source for NO generation truly exists, the group exposed the tomato cell suspensions to various scavengers and inhibitors and demonstrated by fluorescence that the control had the highest amount of fluorescence when compared to the tested concentrations of the scavenger and inhibitors. Additionally the group looked for the presence of a purinergic receptor in the tomato cell suspensions that resembled what occurs in animals. Using ATP, ADP, AMP, ATPγS (an agonist), PPADS (an antagonist), the group demonstrated with fluorescence that ATP

mediates the production of NO using purinergic-like receptors. This paper demonstrates a clear link between the availability of eATP and the increase in NO production.

Because I was germinating *Arabidopsis thaliana* wild-type seeds, I noted that Bethke et al. (2007) offered a review chapter on the role of nitric oxide in seed dormancy and germination. Covering topics such as NO in plant growth and development, challenges in NO chemistry and biology, tools used in NO research, roles of NO and other N-containing compounds in seed dormancy and germination, biochemical and molecular basis of NO action in seeds, this chapter became an excellent resource and background to the role of NO in seed germination. These authors provided a wonderful and concise review of NO synthesis in plants with a great illustration of the interactive mechanisms in the NO signaling pathways. They made clear distinctions between NO synthesis in plant and animal systems yet related the similarities of each making it easy to understand the progression of study from animal systems to plants. They explained clearly how NO is synthesized both enzymatically and non-enzymatically and that those seeds from plants lacking the *AtNOS1* gene are not defective in germination. This chapter raises many questions needing to be explored many of which are currently under examination.

Neill et al. (2008) provide an extensive and thorough review of nitric oxide (NO) generation, removal, perception, movement, and evolution from plants and their work "raises more questions than (it) answers." The authors do a fairly good job describing the paradoxical effects of NO and, in addition, they define many gaps in current knowledge regarding the known NO metabolic pathways. The authors use good figures to illustrate

the summaries of the generation and removal of NO in plant cells. For example, one of the most obvious generalizations that can be made from the illustration describing NO generation and removal (Fig.1) is that NO is generated from arginine in plant organelles such as mitochondria, chloroplasts, peroxisomes, and cytoplasm using the enzyme NO synthase. This illustration also shows four routes of NO generation from NO_2^- occurring in plant sites such as cytoplasm, mitochondria, plasma membrane and chloroplasts, the same areas used in the arginine-generation pathway. Because NO is both lipid and water soluble, NO metabolism would be expected in cellular areas with high lipid concentrations or high water concentrations. These pathways most likely occur simultaneously, but the authors continued to demonstrate that the details of these pathways are unknown. They continued to raise questions regarding "how (plants) use arginine to make NO" and how and where this process is regulated. Another figure illustrates the NO cell map showing where NO is made and removed in cells by several "potential mechanisms and at several intracellular localizations." Given this review, the researcher can narrow the scope of the questions he explores.

Wu and Wu (2008) used a different plant system yet to describe similar effects with the application of exogenous ATP. The authors used a fluorometric method to examine and quantify the presence of NO in hairy root cultures of *Salvia miltiorrhiza* plants exposed to concentrations of ATPγS from 10 to 500 μM and found the optimum concentration for this system to occur at 100 μM. Higher concentrations of ATPγS caused a significant decrease in NO production. Using both ATP and the non-hydrolysable analog of ATP, ATPγS, the authors quantified the similarities between the

two in terms of NO production. The authors continued to examine the dependence of ATP-induced NO production on nitric oxide synthase (NOS), nitrate reductase (NR), Ca^{+2}, and protein kinases finding that ATP induced H_2O_2 production via pathways using Ca^{+2}, protein kinase, and NO biosynthesis. The authors also found that ATP-induced NO production is inhibited by Ca^{+2} antagonists and unaffected by protein kinase inhibitors. Their results demonstrate that NO plays a role in ATP-induced responses and signal transduction in plants. Courtois et al. (2008) provide a review comparing the roles of NO signaling in animal cells with that in plant cells. To help with understanding and summarizing the information, the authors provide two figures, one of an animal cell and one of a plant cell, summarizing the comparison of NO production in relation to Ca^{+2} and protein kinases in both systems. The authors state that very little is known about the signaling proteins involved in plant cells and important goals include identifying "the proteins and investigating how NO modulates their activities…defining the physiological relevance of these modulations and understanding how interplays between NO and Ca^{+2} guide the cell toward a specific response."

Reichler et al. (2008), using treated pollen from *Arabidopsis thaliana* plants of ecotypes Wassilewskija (WS), Columbia (Col-0), and *nia1nia2* mutants and exposing pollen from these ecotypes to 50 μM and 100 μM concentrations ATPγS, found that increasing the extracellular ATPγS, "a poorly hydrolysable version of adenosine nucleotide", would "mimic eATP effects at much lower concentrations." Their results also showed that at concentrations greater than 100μM ATPγS pollen tube germination rates "significantly decreased." Using this analog of ATP prevented the "release of

inorganic phosphate from the applied nucleotides…ensur(ing) the nucleotide signal would be more stable and…more potent than ATP" and allowed the use of a lower concentration of nucleotide to induce the response. Especially interesting to this researcher are the effects of the ATPγS concentrations and the reasons for using the "poorly hydrolysable eATP analog" and the use of the Col-0 ecotype. Because the authors state that "different ecotypes of *Arabidopsis* show significantly different responses to the same stimuli" a natural assumption for this researcher is to expect that the results achieved in pollen germination and pollen tube growth would, or could, be significantly different from those achieved in root hair growth when exposing the root hairs to various concentrations of cGMP, ATPγS, and combinations of the two.

The literature described above was very useful to me both as an introduction into the roles of eATP, and for helping to explain the association between eATP, NO, and cGMP, which is the main focus of my research. Preliminary results in the Roux lab indicated that, although NO inhibits pollen tube growth (Reichler et. al., 2009), it promotes the growth of the root hairs of *Arabidopsis thaliana* Col-0 ecotype. In some systems, NO exerts its signaling effects by activating the enzyme guanylate cyclase to produce more cGMP. In other systems NO exerts its effects by altering the activity of enzymes other than guanylate cyclase, and so these signaling effects of NO are independent of cGMP. The main aim of this research was to discover whether cGMP production was an important intermediate step in mediating the effects of applied nucleotides on root hair growth.

Palavan-Unsal and Arisan (2009) discuss all NO signaling in relation to cGMP, both cGMP-dependence and –independence. They discuss the value of "a cell-permeable analog of cGMP, 8-bromo-cGMP…" as a valuable alternative to another cell-permeable cGMP, dibutyryl cGMP. We decided to use both analogs in my research Additional information on this topic came from Pasqualini et al. (2009) who provided evidence that nitric oxide induces cGMP-dependent and cGMP-independent transcription. The authors note that previous researchers have published results supporting cGMP as a "second messenger in many different physiological responses in higher plants" and its implicated role "in auxin, gibberellic acid and kinetin-dependent signaling." Additionally they note that the time frames for seeing the cytosolic cGMP activity varies from <5 min. to more than 2 hr. depending on the system being examined. The authors note these results as indicators of the complexity and diversity of the signaling roles of cytosolic cGMP in higher plants and suggest that "cGMP has a role as a rapid transducer as well as in sustaining long-term adaptive responses." Summarizing their literature review, the authors state, "while the link between NO and cGMP dependent signaling is well established the biochemical nature of the link remains to be discovered."

All the literature reviewed above provided a strong theoretical background for me to pursue my research on whether cGMP helped to mediate the effects of eATP on root hair growth.

Chapter 3: *Materials, Methods, Analysis and Conclusions*

Plant Material

Colombia ecotype *Arabidopsis thaliana* (Col-0) seeds were first sterilized with a 20% Clorox bleach solution followed by 5 rinses with autoclaved de-ionized water and stored for a minimum of 3 days of vernalization in a cold room at 4^0C in autoclaved de-ionized water. Vernalized seed stocks were used for 2 plantings and then discarded. The seeds were germinated in continuous light for 3-4 days on autoclaved cellophane discs placed on an autoclaved vitamin-enriched Murashige-Skoog agar medium (MS medium) at a pH of ~5.7 in small Petri dishes which were set vertically in the growth chamber. Vitamin stocks are maintained in a -20^0C freezer and are thoroughly thawed and mixed before adding the 100 µL solution to the half-strength MS medium with added sucrose.

Treatment Plates

Fresh MS medium was made the day of treatment and the various treatment concentrations were added to the Petri dishes just prior to adding the agar. Treatment stocks of 20 mM ATPγS and 20 mM dibutyryl cGMP and 20 mM and 2 mM stocks of 8-Br-cGMP are maintained in a constant -40^0C freezer and thawed and mixed thoroughly before use.

Imaging, Measurement, and Analysis of Root Hairs

Seedlings were germinated on cellophane discs that were transferred with forceps from the growth/germination plates to the treatment plates. The transferred seedlings were then photographed at time zero (T=0) and 1 hour later (T=1) using a light microscope set at 40X with a Moticam 2300 camera fitted onto the eyepiece of the

microscope. Motic Images 2.0 software was used to capture the image of each seedling's roots at T=0 and T=1. Capture resolution was set at the largest setting of 2048 X 1536. Image J, a public domain, Java-based imaging program developed by the National Institutes of Health was used to measure root hair lengths on each germinated seedling. Settings for analysis on Image J are as follows: Set scale to distance in pixels = 56, known distance = 100, unit of length = μm, check "Global" and press "Enter". Each picture was zoomed to 75% and each photographed seedling was opened at T=0 and T=1 with Image J. Each germinated seedling root was placed side-by-side (T=0 & T=1) and individual root hairs were matched and measured simultaneously. Raw data was saved in a folder and transferred to a new Excel workbook for data analysis. Average growth rate was calculated by taking the root hair length at T=1, subtracting the root hair length at T=0 and dividing by 60 gave an average growth in μm per minute. Additionally the standard deviation, standard error, and student's t-test (2-tail, type 3) for each treatment and the control were calculated for each experiment. Approximately 2 experiments were done per week.

DATA ANALYSIS AND RESULTS

Previous results investigating the role of cGMP in the eATP signal transduction pathway have been mixed and inconclusive. The goal of this research is to provide more conclusive results on the role of cGMP and to determine whether cGMP is dependent or independent of NO production. Initial experiments were designed to determine a "best concentration of cGMP" in promoting root hair growth. While progressing through this research, we expected to find a typical dose response curve and based on earlier results

(Fig. 1 A and B) that certain concentrations of dibutyryl cGMP would promote growth. However, as seen in Figure 2 we observed inhibition of growth using dibutyryl cGMP. In this particular experiment, the 100 µM concentration was done but the photographed root hairs were too light (no contrast) to be seen or analyzed. Table 1 summarizes the data shown in Figure 1 below. The highlighted cells in the table reflect the data that is significantly different. In this case the root hair growth at the 50 µM concentration is significantly less than that at 10 µM.

Fig. 1A: Data Analysis of Root Hair Growth

	Control	10 µM	25 µM	50 µM
n	22	26	26	37
AVG:	0.740	0.788	0.606	0.514
STDEV:	0.483	0.461	0.408	0.437
S.E.:	0.103	0.090	0.080	0.072
Max:	1.669	1.663	1.387	1.720
Min:	0.054	0.042	0.028	0.060
p-value:		0.728	0.313	0.079
p-value:			0.140	0.021
p-value:				0.392

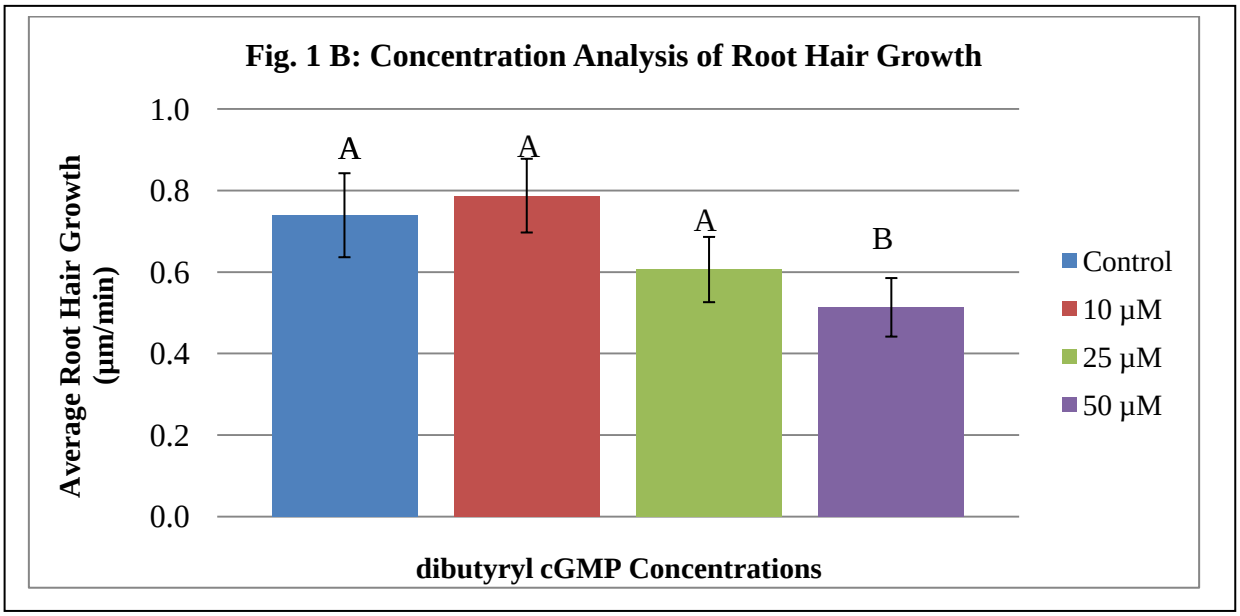


Figure 1: Representative experimental data showing inconsistent and inconclusive results using the non-hydrolysable cGMP analog, dibutyryl cGMP.

The next experiments seemed to show that the best root hair growth occurred when using 25 µM concentrations of dibutyryl cGMP which was quite unexpected. Table 2 summarizes the data from experiment 5 and Figure 2 is representative of the data. This data shows a significant increase in root hair growth between the smallest concentration (1 µM) and the 25 µM concentration of dibutyryl cGMP. But it also shows no significant differences in root hair growth when the different concentrations are compared to the control, except in the 10 µM.

Fig. 2 A: Data Analysis of Root Hair Growth					
	Control	1 µM	10 µM	25 µM	50 µM
n	73	53	53	31	36
AVG:	0.884	0.653	0.924	1.083	0.929
STDEV:	0.514	0.476	0.469	0.770	0.575
S.E.:	0.060	0.065	0.064	0.138	0.096
Max:	2.704	2.320	1.673	3.690	2.387
Min:	0.077	0.047	0.030	0.111	0.025
p-value:		0.010	0.653	0.195	0.692
p-value:			0.004	0.007	0.020
p-value:				0.303	0.964
p-value:					0.365

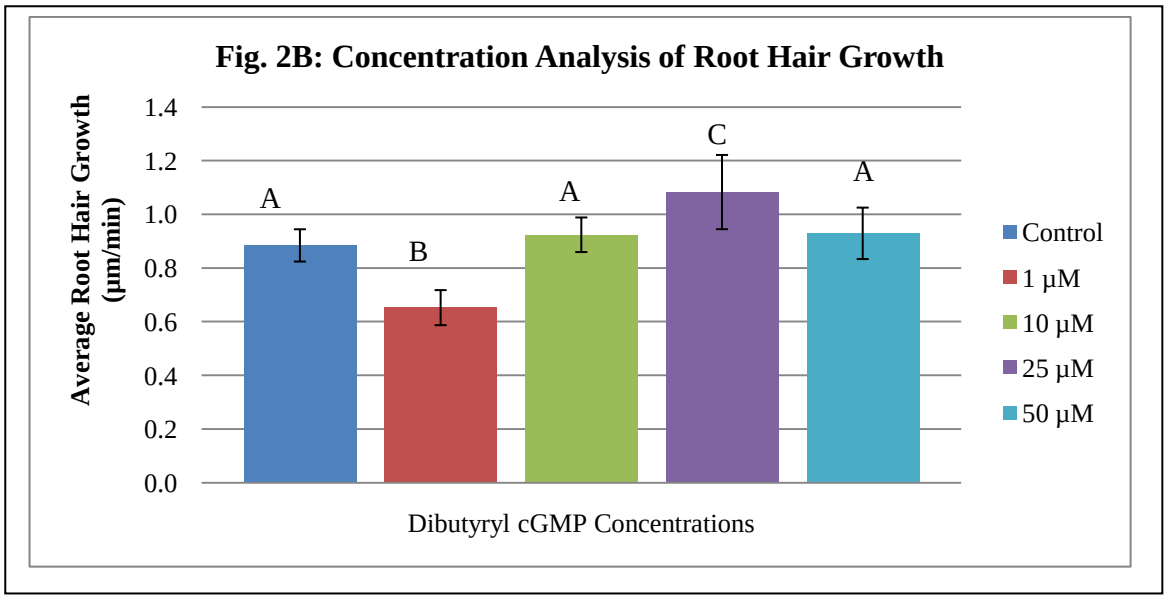


Figure 2: Representative experimental data showing inconsistent and inconclusive results using the non-hydrolysable cGMP analog, dibutyryl cGMP.

Overall the results using dibutyryl cGMP over six experiments continued to be inconsistent and inconclusive, and upon further literature review we found that there was another "cell-permeable analogue of cGMP (8-bromo-cGMP)" which relieved the "NO-induced programmed cell death (which) was inhibited by ODQ." (Palavan-Unsal and Arisan, 2009) The use of this different analog became intriguing as a potential answer to

whether there is a better "non-hydrolysable analog" for cGMP which positively influences root hair growth. Once acquired, the 8-bromo-cGMP was used in successive experiments seeking a best concentration of 8-Br-cGMP to positively affect root hair growth and a reversal of inhibition of root hair growth by ATPγS concurrently. Figure 3 A summarizes the data analysis for the first experiment done using the 8-bromo-cGMP and Figure 3 B shows the graphic results.

Fig. 3 A: Data Analysis of Root Hair Growth using 8-Br-cGMP & ATPγS

	Control	10 µM 8-Br-cGMP	100 µM 8-Br-cGMP	150 µM ATPγS	150 µM ATPγS + 10 µM 8-Br-cGMP
n	81	83	45	23	60
AVG:	0.711	0.892	1.042	0.793	0.883
STDEV:	0.406	0.448	0.500	0.563	0.526
S.E.:	0.045	0.049	0.075	0.117	0.068
Max:	1.983	2.053	1.865	2.360	1.829
Min:	0.031	0.038	0.060	0.037	0.039
p-value:		0.008	2.982E-04	0.521	0.038
p-value:			0.097	0.443	0.914
p-value:				0.081	0.118
p-value:					0.511

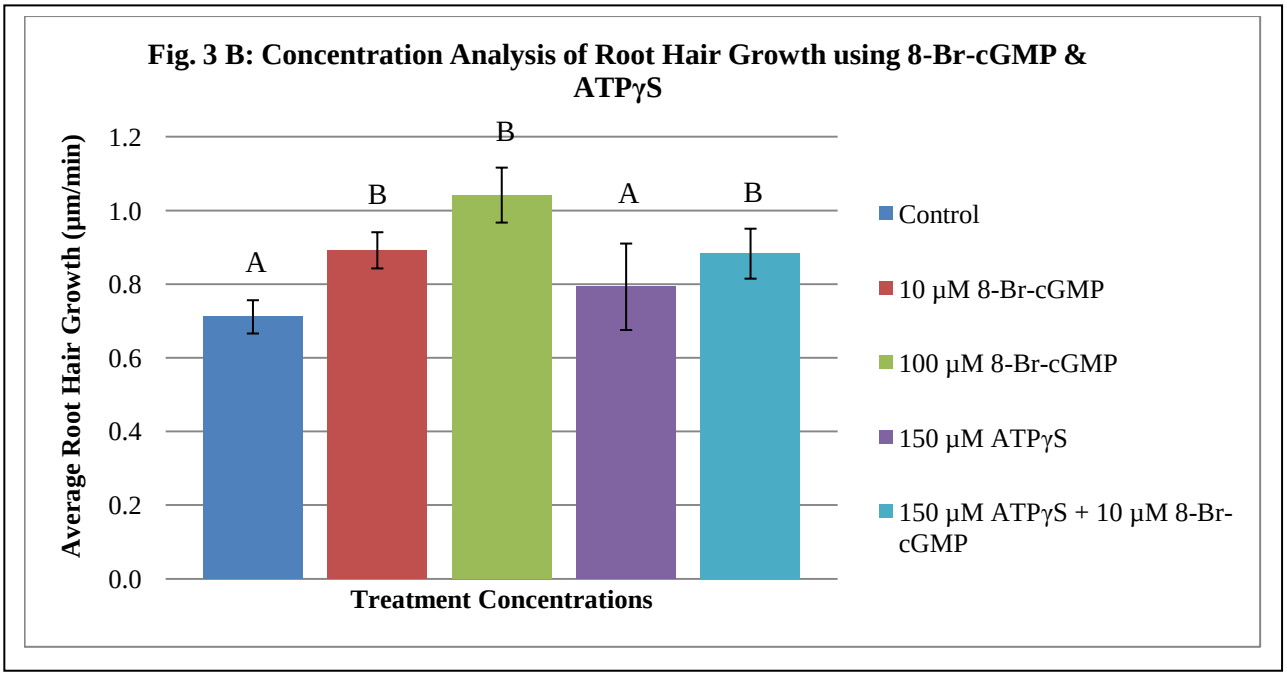


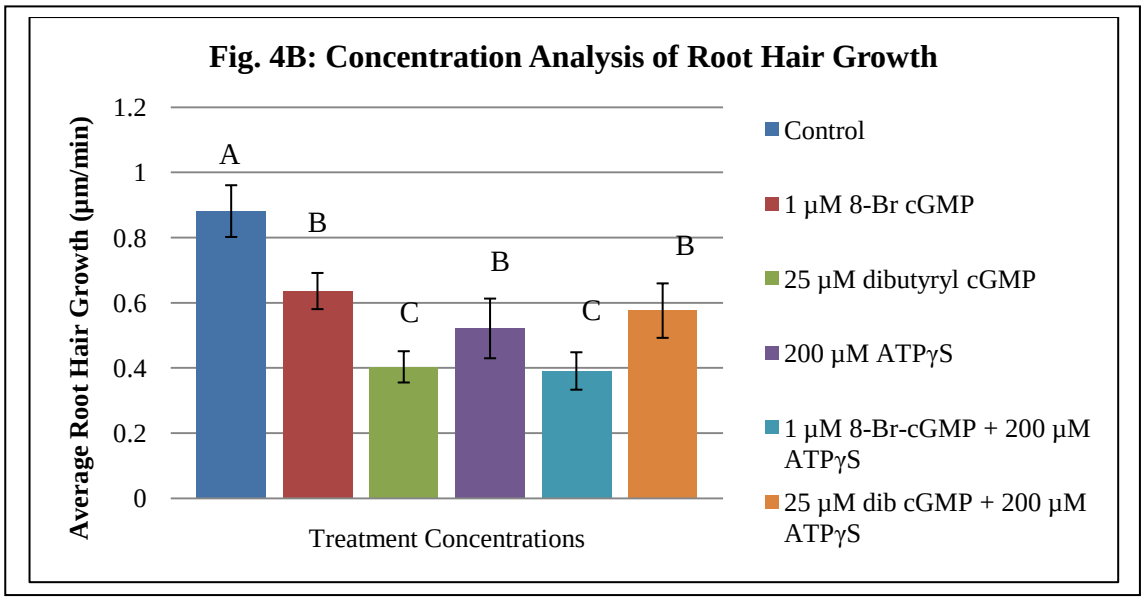
Figure 3: Representative experimental data showing inconsistent and inconclusive results using the non-hydrolysable cGMP analog, dibutyryl cGMP.

Seeing a better and more definite dose response curve with the 8-Br-cGMP has resulted in the abandonment of the dibutyryl c-GMP in favor of the 8-Br-cGMP. Also, observing the lowered average growth rate under the influence of the 150 µM concentrations of ATPγS alone followed by the raise in the average growth rate in root hairs exposed to the combination of 150 µM ATPγS and 10 µM 8-Br-cGMP has encouraged the use of 8-Br-cGMP and a higher concentration of ATPγS (200 µM) in subsequent experiments.

The final experiment pitted the two cGMP analogs, which in previous experiments had promoted root hair growth the best, against each other and in combination with a high concentration of ATPγS. The hypothesis for this final experiment was that the 8-Br-cGMP analog would promote root hair growth better than

its counterpart, dibutyryl cGMP. We also expected to see the inhibition of root hair growth with exposure to the 200 µM ATPγS. The results are given below in Fig. 4.

	Fig 4A: Data Analysis of Root Hair Growth					
	Control	1 µM 8-Br cGMP	25 µM dibutyryl cGMP	200 µM ATPγS	1 µM 8-Br-cGMP + 200 µM ATPγS	25 µM dib cGMP + 200 µM ATPγS
n:	60	78	57	29	45	31
AVG:	0.881	0.636	0.403	0.521	0.390	0.576
STDEV:	0.615	0.490	0.362	0.493	0.385	0.465
S.E.:	0.079	0.055	0.048	0.092	0.057	0.084
MIN:	0.074	0.0421	0.046	0.0356	0.034	0.042
MAX:	3.03	1.927	1.262	1.59	1.455	1.933
p-value:		0.013	1.357E-06	0.004	1.102E-10	0.010
p-value:			0.002	0.289	0.003	0.551
p-value:				0.260	0.866	0.079
p-value:					0.233	0.661
p-value:						0.056



These data show again that there is no clear, concise, or consistent root hair growth when root hairs are exposed to concentrations of cGMP. However, the data also

show that the 8-Br-cGMP analog promotes root hair growth better than the dibutyryl cGMP analog. These data also show that ATPγS can inhibit root hair growth as has been previously found in the Roux lab (unpublished results).

Conclusions

Because neither cGMP analog consistently promoted or inhibited root hair growth at any concentration, a tentative conclusion is that the effects of NO on root hair growth are being mediated in a cGMP-independent fashion. These experiments should be repeated by other experimenters to validate these preliminary results. Exposing root hairs to a NO donor, such as NONOate or SNAP, in the presence of the non-hydrolyzable analogs of cGMP may give some additional insight into the relationship between NO and cGMP. Also using ODQ as an NO scavenger or competitor for guanylate cyclase in the presence of these non-hydrolyzable cGMP analogs may provide additional insights into this intermediate relationship. Because Neill et al. (2008) state that "…diffusion of NO within the plant may be relatively restricted and there might exist 'NO hot-spots' depending on the sites of NO generation and the local biochemical micro-environment", narrowing the sites of NO generation may provide direction for subsequent studies. Of course, manipulating the *Arabidopsis* cGMP-dependent transcriptome mentioned by Pasqualini et al. (2009) would provide more information about the mediating role of cGMP on root hair growth. These authors state that "it remains unclear how NO is synthesized *in planta*" and maybe going back to this early step in the eATP signaling pathway to root hair growth would be a beneficial investigation. Needless to say, the jury is still out on the exact role cGMP plays in mediating the effects of NO on root hair growth in *Arabidopsis thaliana*. However, with more experimentation I am certain the jury will receive the information they need to make a significant verdict.

Chapter 4: *Applications to Practice*

Arabidopsis thaliana is a model plant that plays a role in agriculture which is similar to the roles played by fruit flies and mice in animal biology. With a genome of about 157 million base pairs on five chromosomes with 27,000 genes expressing about 35,000 proteins, *Arabidopsis thaliana* was the first plant to be sequenced. Sequencing information is kept in The Arabidopsis Information Resource (TAIR), the Arabidopsis Biological Resource Center (ABRC), and other places around the United States and the world. Growth and reproduction studies sponsored by the European Space Agency on *Arabidopsis thaliana* are on-going aboard the International Space Station.

As a model organism, *Arabidopsis thaliana* is well-suited for study using light microscopy. Because *Arabidopsis* roots are relatively translucent, they lend themselves to easy live cell studies and imaging. Because *Arabidopsis thaliana* plants can go from seed-to-seed in 60 days and from seed-to-flower in 21 days, and because their flowers are self-pollinating, they would be easy to grow and study in a classroom laboratory setting. Because *Arabidopsis thaliana* is a member of the Brassicaceae family of plants and a relative to broccoli, cauliflower, cabbage, and other similar plants, I would be interested in growing these common vegetables in the classroom and comparing them to *Arabidopsis thaliana* plants.

Performing experiments can be a difficult undertaking in a non-traditional high school setting, such as Harrell Accelerated Learning Center. However, I believe that working with plants poses much less concern for the students in my classes. Typically, these students are very bright, but because of their lack of motivation to attend school, they have fallen behind their classmates. In many cases these students have been court ordered to attend school, pass their GED, and complete the requirements for their high

school diplomas. In other cases these students have had negative experiences in their science classes and come into my science class thinking they can not "do science." These students are often in my classes for less than a six-week grading period, so *Arabidopsis* studies will help students have a quick, easy, and maybe less threatening organism to study. Also because I have already done twelve experiments and have the raw data available, these students can gain quick, easy, and less threatening lessons in data analysis.

One of the major obstacles to student achievement in my scenario is the fact that many students fail to pass the high stakes test for graduation, Texas Assessment of Knowledge and Skills, or TAKS. Having data already available for student analysis eliminates the time it takes to do an experiment and allows students the experience of using raw data, constructing meaningful data tables, using computer programs to analyze data, and reporting meaningful conclusions, all of which are covered on the TAKS test. Using a traditional light microscope with a camera attached is a great way to demonstrate the importance of knowing the parts of a microscope and how to safely use one. Also use of the light microscope in my experiments shows students that what they are learning in high school is used in "real life" situations. Relating the different types of technology I used in these experiments also shows my students that there are many scientific careers in which computer-aided technology is used and in every research group, a person with those skills is priceless.

Another lesson my students can learn from the experience I have had is that nothing is fast in research. Students in my classes want the right answer immediately and do not want to take the time to even look up the information they need to draw a valid conclusion or to make a valid inference. Setting up an experiment so that one variable is tested is a difficult concept for my students to grasp. With *Arabidopsis thaliana* being a

model organism and easy to grow, bloom, and study, setting up experiments for my students to do in class will be an excellent way to introduce and use the scientific method while teaching them that nothing is fast in research and often your results are not what you originally hypothesized. My students interpret the "failure to get what you hypothesize" as a failure which makes science "hard." Maybe using these little plants in the classroom and doing several experiments will teach my students that science is not "hard" but that every experiment provides information not previously known by the researcher.

Another important use of this research in my classes will be the calculations used during the data analysis portion of my study. Math and science are tightly linked and my students fear both. Using math to calculate concentrations for treatments as well as calculating root hair growth in microns per minute (μm/min) are excellent ways to show students that what they learn in their math classes is transferable to other academic areas and it is important to the success of their research. Discussions of the data and whether the data is "significant" are important extensions of the data analysis experience. Without going into great detail on statistics, but still using the computer-aided tools available to students in my classroom, students will become more comfortable using math to analyze claims they see on television, or read in the newspaper. These experiences will give my students the ability to analyze the validity of these claims and make better purchasing decisions. Again this is another objective tested heavily on the TAKS.

Once students have done a few experiments with their *Arabidopsis thaliana* plants, have analyzed their results, made valid conclusions based on those results, students will have the opportunity to express their experience in a report. Prior to writing this report, the students will have the opportunity to read and evaluate a published article

from a scientific journal regarding what facet of *Arabidopsis thaliana* growth and development they are studying. This activity provides students exposure to previously published scientific literature, allows students to build their vocabulary, and learn what they need to include in such a report for their own research. Communication, whether by chemical signaling in plants or by verbal signaling on paper, is one of the most important activities with which students are involved. Learning to communicate effectively is a skill that my students will use for the remainder of their lives.

I want to begin my evaluation of the Master of Arts in Science and Mathematics Education in the UTeach program here at the University of Texas Austin by saying that the experience has been wonderful. I have enjoyed almost everything I have done and I have truly enjoyed everything I have learned. The education classes were informative and allowed time to apply the concepts to the real world of high school science. The science classes were excellent in allowing me time to get into recent advances in scientific research and explore topics that are difficult to study and learn and yet apply to my non-traditional classroom. As I stated earlier, WOW!!! Having worked previously in a masters program I found this experience to be exciting and enlightening. I have enjoyed every aspect of the classes offered and have found them engaging and challenging. I have learned a great deal about ways to improve my craft of teaching and educating today's youth. I have learned that it's not nearly as important that you know, but that you know how to think, how to reason, how to explore, and how to draw valid conclusions based on the experimental results in front of you. I have had the greatest amount of frustration and joy, sometimes almost within the same moment, while working on my final project. I love science because the possibilities are endless and uncertain. Every experiment done and results analyzed gives new insights into your unanswered questions. How exciting is that!!! If we educators could teach science purely through

original research we would, and could, turn our unmotivated, unchallenged, classroom discipline problems (you teachers know their names) into tomorrow's scientists. I believe one of the main goals of the Freshman Research Initiative here at the University of Texas is to take incoming freshman and expose them to original research. I would like to see something similar started in the high schools in my neck of the woods and through the UTeach masters program I have gained the tools to launch such a program, even if it only begins in my classroom. Thank you so much, Dr. Ruth Buskirk & Dr. Mary Walker, for giving me this opportunity!!! Thank you so much Dr. Stan Roux and Dr. Greg Clark for taking me under your wings and making this project happen!!!

Annotated Bibliography

Bethke, P. C., Libourel, I. G. L., and Jones, R. 2007. Nitric oxide in seed dormancy and germination. Seed Development, Dormancy, and Germination. Edited by Bradford, K. J. and Nonogaki, H. Ames: Blackwell Publishing, Ltd.

Because I was germinating *Arabidopsis thaliana* wild-type seeds, these authors offered a review of the role of nitric oxide in seed dormancy and germination. Covering topics such as NO in plant growth and development, challenges in NO chemistry and biology, tools used in NO research, roles of NO and other N-containing compounds in seed dormancy and germination, biochemical and molecular basis of NO action in seeds, interactions between NO and phytochrome or ABA, ecological significance of NO, this chapter became an excellent resource and background to the role of NO in seed germination. These authors provided a wonderful and concise review of NO synthesis in plants with a great illustration of the interactive mechanisms in the NO signaling pathways. They made clear distinctions between NO synthesis in plant and animal systems yet related the similarities of each making it easy to understand the progression of study from animal systems to plants. They explained clearly how NO is synthesized both enzymatically and non-enzymatically and that those seeds from plants lacking the *AtNOS1* gene are not defective in germination. This chapter raises many questions needing to be explored many of which are currently under examination.

Clark, G.B., Thompson, Jr., G., and Roux, S.J. 2001. Signal Transduction mechanisms in plants: An overview. *Current Science, Vol. 80, No. 2, 25 January 2001, pp. 170-177.*

This article provides an excellent starting point for a researcher interested in exploring the various signal transduction mechanisms in plants. The article reviews previously published literature covering the mechanisms of calcium-based signaling, including discussions of the measuring and regulation of Ca^{+2} in cells, the targets of Ca^{+2} signals and Ca^{+2}-activated proteins, CaM (calmodulin), annexins, and protein kinase C. Additionally, the authors continue their discussion of plant signaling mechanisms with a discussion of the mechanisms involving inositol phospholipids and G protein-mediated signaling. All the mechanisms reviewed are very complex, working in multi-layered networks and never in isolation from other signaling pathways, and affect all aspects of plant growth and development. The authors do include a discussion of recently published findings on three extracellular agents in these signaling pathways: extracellular CaM, extracellular ATP, and integrin-like receptors. The article summarizes well the research published previous to January 2001. Almost ten years later new knowledge has been gained, but this field continues to be wide open in terms of understanding the complex signaling pathways in plants.

Courtois, C., Besson, A., Dahan, J., Bourque, S., Dobrowolska, G., Pugin, A., and Wendehenne, D. 2008. Nitric oxide signaling in plants: interplays with Ca^{+2} and protein kinases. *Journal of Experimental Botany, Vol. 59, No. 2, pp. 155-163.*

This paper provides a review comparing the roles of NO signaling in animal cells with that in plant cells. To help with understanding and summarizing the information, the authors provide two figures, one of an animal cell and one of a plant cell, summarizing the comparison of NO production in relation to Ca^{+2} and protein kinases in both systems. The authors state that very little is known about the signaling proteins involved in plant cells and important goals include identifying "the proteins and investigating how NO modulates their activities…defining the physiological relevance of these modulations and understanding how interplays between NO and Ca^{+2} guide the cell toward a specific response."

Demidichik, V., Nichols, C., Oliynyk, M., Dark, A., Glover, B.J., and Davies, J.M. 2003. Is ATP a Signaling Agent in Plants? *Plant Physiology, October 2003, Vol. 133, pp. 456-461.*

The objective of this study was to investigate possible eATP-induced signaling pathways. The authors hypothesized that , because Ca^{+2} is a downstream second messenger from eATP in animal cells, the eATP would have no effect on cytosolic calcium ion concentrations in *Arabidopsis thaliana* plants with excised roots (to prevent eATP exposure from soil microorganisms or root hair damage). Experimental procedures are described in a previously published paper, but Figure 1 shows the effect of various externally applied purines and UTP on *Arabidopsis* root $[Ca^{+2}]_{cyt}$ in a dose-response curve with various agonists on the $[Ca^{+2}]_{cyt}$. The study disproved the null hypothesis meaning that in root cells eATP can elevate $[Ca^{+2}]_{cyt}$.

Foresi, N.P., Laxalt, A.M., Tonón, C.V., Casalongué, C.A., and Lamattina, L. 2007. Extracellular ATP Induces Nitric Oxide Production in Tomato Cell Suspensions. *Plant Physiology, November 2007, Vol.145, pp. 589-592.*

This scientific correspondence shows the results of several experiments in the study of the effect of exogenous ATP on nitric oxide (NO) production in tomato cell suspensions. Using fluorescence and a microwell fluorometer over a 2-hr. time period, this group demonstrates that 1mM concentrations of ATP generated high levels of NO as expressed in arbitrary units (AU) of green fluorescence. The group continued their study by using an ATP concentration gradient and demonstrated an increase in NO generation (as expressed with fluorescence) with an increase in ATP concentration. Also remarkable is the fact that the cell suspensions with no ATP exposure incurred a slight increase in NO generation, thus demonstrating a cellular source of minimal NO production. To insure that a cellular source for NO generation truly exists, the group exposed the tomato cell suspensions to various scavengers and inhibitors and demonstrated by

fluorescence that the control had the highest amount of fluorescence when compared to the tested concentrations of the scavenger and inhibitors. Additionally the group looked for the presence of a purinergic receptor in the tomato cell suspensions that resembled what occurs in animals. Using ATP, ADP, AMP, ATPγS (an agonist), PPADS (an antagonist), the group demonstrated with fluorescence that ATP mediates the production of NO using purinergic-like receptors. This paper demonstrates a clear link between the availability of eATP and the increase in NO production.

Kim, S-Y., Sivaguru, M., and Stacey, G. 2006. Extracellular ATP in Plants. Visualization, Localization, and Analysis of Physiological Significance in Growth and Signaling. *Plant Physiology, November 2006, Vol.142, pp. 984-992.*

This remarkable study about extracellular ATP (eATP) provides "definitive experimental evidence for (the) presence or (the) explanation as to how…a polar molecule could exit the plant cell and what physiological role it may play in plant growth and development." Relating the well-documented evidence of eATP in animal systems, the authors devised a novel reporter linking the "ATP-requiring enzyme luciferase" to a cellulose-binding domain peptide and used the reporter to allow "visualization of eATP in the presence of the substrate luciferin." Luciferase activity was noted in the extracellular matrix (ECM) between epidermal cells in actively growing roots of *Medicago trunculata* plants. Pre-treating the seedlings in water for one hour with several pharmacological agents such as a nonhydrolyzable ATP, potato apyrase, $GdCl_3$, BAPTA, $LaCl_3$, and brefeldin A allowed the authors to demonstrate that ATP is prevalent at root tips and actively growing areas of the plant, that the CBD-Luciferase protein complex is distributed evenly at root hair surfaces and is associated strongly with the cell wall, that ATP secretion is calcium dependent, that ATP plays a role in the reactive oxygen species (ROS) in root hair tips, and that this enhanced eATP secretion is found in actively growing epidermal and cortical cells of etiolated hypocotyls and in the zone of elongation in root apices yet is minimal in the more mature areas of roots. This landmark study proves unequivocally that eATP is a central player in plant cell signal transduction pathways.

Lamotte, O., Courtois, C., Barnavon, L., Pugin, A., and Wendehenne, D. 2005. Nitric oxide in plants: the biosynthesis and cell signaling properties of a fascinating molecule. *Planta (2005) 221: 1-4.*

This progress report has a great schematic representation of the NO signalling pathways in plant cells showing all the routes for NO synthesis. The authors also provide a short description of several questions that needed to be answered at the time of their report to include a study of the chemistry involved in the reaction catalysed by NOS, understanding the mechanisms of the NO signaling pathways by studying the protein, AtNOS1, its location in plant cells, and other proteins with which it may be involved, a study of the *AtNOS1* gene, its regulation and

expression in the growth and development of plants and its role in the environmental responses of plants. Lastly the authors think a study into the identification of the NO targets in plants would be beneficial.

Lew, R.R., and Dearnaley, J.D.W. 2000. Extracellular nucleotide effects on the electrical properties of growing *Arabidopsis thaliana* root hairs. *Plant Science 153 (1): 1-6.*

These authors devised a procedure to explore the membrane potentials and the nucleotides that caused them. The authors found that the nucleotides, ATP and ADP, depolarized the plasma membrane of *Arabidopsis thaliana* root hairs and that the "relative effectiveness of… nucleotides was ATP=ADP=GTP>GMP>AMP>TTP(=adenosine)>CTP." They also learned that phosphates had no effect on membrane potentials indicating that the nucleotide effects were not due to the hydrolysis and uptake of phosphates. Because the authors found that ADP had a high specificity effect on the membrane potentials, they suggested a role for ADP as an "extracellular message…as a signal during cellular wounding…"

Neill, S., Bright, J., Desikan, R., Hancock, J., Harrison, J., and Wilson, I. 2008. Nitric oxide evolution and perception. *Journal of Experimental Botany, Vol. 59, No. 1, pp. 25-35.*

This extensive and thorough review of nitric oxide (NO) generation, removal, perception, movement, and evolution from plants "raises more questions than (it) answers." The authors do a fairly good job describing the paradoxical effects of NO and, in addition, they define many gaps in current knowledge regarding the known NO metabolic pathways. The authors use good figures to illustrate the summaries of the generation and removal of NO in plant cells. For example, one of the most obvious generalizations that can be made from the illustration describing NO generation and removal (Fig.1) is that NO is generated from arginine in plant organelles such as mitochondria, chloroplasts, peroxisomes, and cytoplasm using the enzyme NO synthase. This illustration also shows four routes of NO generation from NO_2^- occurring in plant sites such as cytoplasm, mitochondria, plasma membrane and chloroplasts, the same areas used in the arginine-generation pathway. Because NO is both lipid and water soluble, NO metabolism would be expected in cellular areas with high lipid concentrations or high water concentrations. These pathways most likely occur simultaneously, but the authors continued to demonstrate that the details of these pathways are unknown. They continued to raise questions regarding "how (plants) use arginine to make NO" and how and where this process is regulated. Another figure illustrates the NO cell map showing where NO is made and removed in cells by several "potential mechanisms and at several intracellular localizations." Given this review, the researcher can narrow the scope of the questions he explores.

Pasqualini, S., Meier, S, Gehring, C., Madeo, L., Fornaciari, M., Romano, B, and Ederli, L. 2009. Ozone and nitric oxide induce cGMP-dependent and –independent transcription of defence genes in tobacco. *New Phytologist (2009) 181: 860-870.*

The interesting part of this paper is the claim that nitric oxide induces cGMP-dependent and cGMP-independent transcription. The authors note that previous researchers have published results supporting cGMP as a "second messenger in many different physiological responses in higher plants" and its implicated role "in auxin, gibberellic acid and kinetin-dependent signaling." Additionally they note that the time frames for seeing the cytosolic cGMP activity varies from <5 min. to more than 2 hr. depending on the system being examined. The authors note these results as indicators of the complexity and diversity of the signaling roles of cytosolic cGMP in higher plants and suggest that "cGMP has a role as a rapid transducer as well as in sustaining long-term adaptive responses." Summarizing their literature review, the authors state, "while the link between NO and cGMP dependent signaling is well established the biochemical nature of the link remains to be discovered." Because their study investigates the presence of defence genes in tobacco plants, my interest in this article was limited to the portion of the title and introductory paragraphs stating the roles of nitric acid inducing cGMP-dependent and –independent responses.

Palavan-Unsal, N. and Arisan, D. 2009. Nitric Oxide Signalling in Plants. *Botanical Review (2009) 75: 203-229.*

This review discusses the all the facets involved in the recent advances NO synthesis and the function of NO signaling in plants. Figures show the different synthetic pathways for NO, different NO signaling mechanisms found in plants, and the NO signal transduction pathway. A table summarizes the effects of NO on plant growth, development, and its effect on plant growth hormones and regulators. Of great interest to this researcher was the section which discusses the NO signaling relation with cGMP, both cGMP-dependence and –independence. The interesting portion of this discussion was the mention of "a cell-permeable analog of cGMP, 8-bromo-cGMP…" This seems to be a different analog of cGMP from what I am using in my research.

Reichler, S., Torres, J., Rivera, A., Cintolesi, V.A., Clark, G., and Roux, S.J. 2009. Intersection of two signaling pathways: extracellular nucleotides regulate pollen germination and pollen tube growth via nitric oxide. *Journal of Experimental Botany, Vol. 60, No. 7, pp. 2129-2138.*

Using treated pollen from *Arabidopsis thaliana* plants of ecotypes Wassilewskija (WS), Columbia (Col-0), and *nia1nia2* mutants and exposing pollen from these ecotypes to 50 μM and 100 μM concentrations ATPγS, these authors found that when they increased the extracellular ATPγS, "a poorly hydrolysable version of adenosine nucleotide", would "mimic eATP effects at much lower concentrations." Their results also showed that at concentrations greater than

100μM ATPγS pollen tube germination rates "significantly decreased." Using this analog of ATP prevented the "release of inorganic phosphate from the applied nucleotides…ensur(ing) the nucleotide signal would be more stable and…more potent than ATP" and allowed the use of a lower concentration of nucleotide to induce the response. Especially interesting to this researcher are the effects of the ATPγS concentrations and the reasons for using the "poorly hydrolysable eATP analog" and the use of the Col-0 ecotype. Because the authors state that "different ecotypes of *Arabidopsis* show significantly different responses to the same stimuli" a natural assumption for this researcher is to expect that the results achieved in pollen germination and pollen tube growth would, or could, be significantly different from those achieved in root hair growth when exposing the root hairs to various concentrations of cGMP, ATPγS, and combinations of the two. Therefore, I am unsure as to whether the outcomes would be similar when applied to the root hairs of *Arabidopsis thaliana* Col-0 ecotype. I would like to investigate the effects of adding an NO donor, such as SNAP or NONOate, or using the ODQ inhibitor of guanylate cyclase, to ensure that enough NO is present in the system to achieve significant root hair growth. It may be necessary to ensure adequate NOS and NR quantities are present in the system to achieve consistent stimulation of root hair growth.

Riewe, D., Grosman, L., Fernie, A.R., Wucke, C., Geigenberger, P. 2008. The Potato-Specific Apyrase Is Apoplastically Localized and Has Influence on Gene Expression, Growth, and Development. *Plant Physiology, July 2008, Vol. 147, pp. 1092-1109.*

Using reverse genetic and biochemical approaches, these authors investigate the role of potato-specific apyrase. Silencing the apyrase gene family resulted in many phenotypic changes in the transgenic lines to include "a general retardation in growth, an increase in tuber number per plant, and differences in tuber morphology." These authors demonstrated the role of the enzyme in the apoplast, and provide evidence for its direct involvement in regulating growth and development in plants.

Roux, S.J., Thomas, C., and Rajagopal, A. 2001. A Role for Ectoapyrases and Extracellular ATP in Plant Growth and Development. *Signal Transduction in Plants: Current Advances.* Edited by Sopory, S.K., Oelmüller, R. and Maheshwari, S.C. Kluwer Academic/Plenum Publishers. New York, New York.

This chapter provides an introduction to the role apyrases play in hydrolyzing the γ and β phosphates on the ATP molecule and the last phosphate on ADP. Due to the fact that these enzymes are located in the plasma membrane with their active sites facing into the ECM they are called ectoapyrases. The chapter immediately defines the apyrase class of enzymes and then answers the question of how ATP leaves the cells. The authors provide three exit strategies for eATP's presence in the ECM. First, eATP leaves the plant cells when the cells undergo autolysis, or

self-destruction. Second, eATP leaves the plant cells when the cell's vesicles merge into the plasma membrane and the vesicle contents are then dumped into the ECM. Lastly, the eATP leaves the cell through specialized transport proteins, such as the ABC transporters which mediate either directly or indirectly the intracellular adenylate accumulations involved in signaling mechanisms. The chapter continues to describe this final exit mode and explores the activity of these enzymes. The authors continue to relate the ectoapyrase activity in animal systems to plants. The best part of this chapter is the clear and concise presentation of laboratory studies published and non-published relating to the effects of the apyrases. The authors continue their exploration into the role of the MDR1 and the ectoapyrase in toxin resistance. This answers my question as to why we use a non-hydrolyzable analog of cGMP. This nonhydrolyzable analog is immune to the effects of the apyrases present in the ECM.

Roux, S.J. and Steinebrunner, I. 2007. Extracellular ATP: an unexpected role as a signaler in plants. *Trends in Plant Science, Vol. 12, No. 11, 2007, pp. 522-527.*

This article is one of the first I read authored by my research supervisor. A very concise and easily understood primer describing the roles of extracellular ATP (eATP) as a signaling molecule in plants, the article raises questions, reviews potential answers published by various researchers, reviews literature describing similarities between eATP signaling and responses seen in animals and plants, and provides a direction for future research. However, this review poses no direct association between eATP, NO, and cGMP, which is the basis for my research. This article is very useful as an introduction into the roles of eATP. My research is exploring the roles of cGMP in the complex pathway between eATP and the stimulation of root hair growth.

Stöhr, C., Strube, F., Marx, G., Ulrich, W.R., and Rockel, P. 2001. A plasma membrane-bound enzyme of tobacco roots catalyses the formation of nitric oxide from nitrite. *Planta (2001) 212: 835-841.*

These researchers found "nitrite-reducing enzyme activity that resulted in nitric oxide (NO) formation" in the purified plasma membranes of tobacco roots. This enzyme activity was not found in the plasma membrane vesicles and protein fractions of leaves. This paper reveals another possible explanation for the presence of NO in plant roots. This group also separated the two enzymes in question, PM-NR (plasma membrane nitrate reductase) and NI-NOR (nitrite: NO-reductase) with a western blot analysis.

Song, C.J., Steinebrunner, I., Wang, X., Stout, S.C., and Roux, S.J. 2006. Extracellular ATP Induces the Accumulation of Superoxide via NADPH Oxidases in *Arabidopsis*. *Plant Physiology, April 2006, Vol. 140, pp. 1222-1232.*

The authors report in this study the support of their hypothesis that, as in animal cells where eATP induces the production of reactive oxygen species (ROS)

through the mediation of NADPH oxidase, in leaves of *Arabidopsis* applied ATP induced the "accumulation of superoxide (O_2^-) in a dose-dependent manner.

Wolf, C., Hennig, M., Romanovicz, D., Steinebrunner, I. 2007. Developmental defects and seedling lethality in apyrase *AtAPY1* and *AtAPY2* double knockout mutants. *Plant Molecular Biology (2007) 64:657-672.*

This study uses a double knockout technique to restore pollen germination and generate "apyrase T-DNA double knockouts (DKO) *apy1-1/apy1-1; apy2-1/apy2-1* by complementation with *AtAPY2* under the control of a pollen-specific promoter." The DKO plants produced had many developmental defects including the lack of functioning root and shoot meristems and unlobed pavement cells and stomatal clusters. The addition of a dexamethasone-(DEX)-inducible promoter produced less severe types of developmental defects in plant cells, but these mutants were also seedling lethal. These authors demonstrated the importance of the *AtAPY 1* and *AtAPY2* genes in normal plant development.

Wu, J., Steinebrunner, I., Sun, Y., Butterfield, T., Torres, J., Arnold, D., Gonzalez, A., Jacob, F., Reichler, S., and Roux, S.J. 2007. Apyrases (Nucleoside Triphosphate-Diphosphohydrolases) play a key role in growth control in *Arabidopsis*. *Plant Physiology, June 2007, Vol. 144, pp. 961-975.*

These authors report the highest expression of apyrases in actively growing tissues in *Arabidopsis.* Two genes with a high degree of similarity between them, *APY1* and *APY2*, were analyzed using β – glucuronidase during seedling development under various light treatments. The authors state "(t)hese results imply that APY1 and APY2, like their homologs in animals, act to reduce the concentration of extracellular nucleotides, and that this function is important for the regulation of growth in Arabidopsis."

Wu, S-J. and Wu, J-Y. 2008. Extracellular ATP-induced NO production and its dependence on membrane Ca^{+2} flux in *Salvia miltiorrhiza* hairy roots. *Journal of Experimental Botany, Vol. 59, No. 14, pp. 4007-4016.*

This paper uses a different plant system yet describes similar effects with the application of exogenous ATP. The authors used a fluorometric method to examine and quantify the presence of NO in hairy root cultures of *Salvia miltiorrhiza* plants exposed to concentrations of ATPγS from 10 to 500 μM and found the optimum concentration for this system to occur at 100 μM. Higher concentrations of ATPγS caused a significant decrease in NO production. Using both ATP and the non-hydrolysable analog of ATP, ATPγS, the authors quantified the similarities between the two in terms of NO production. The authors continued to examine the dependence of ATP-induced NO production on nitric oxide synthase (NOS), nitrate reductase (NR), Ca^{+2}, and protein kinases finding that ATP induced H_2O_2 production via pathways using Ca^{+2}, protein kinase, and NO biosynthesis. The authors also found that ATP-induced NO production is inhibited by Ca^{+2} antagonists and unaffected by protein kinase

inhibitors. Their results demonstrate that NO plays a role in ATP-induced responses and signal transduction in plants.

Yamasaki, H. 2005. The NO world for plants: achieving balance in an open system. *Plant, Cell and Environment (2005) 28: 78-84.*

This paper compares the two nitric oxide (NO) production pathways in plants: the arginine pathway (the more complex path where oxygen and enzymes are required) and the nitrite pathway (the simpler path where chemicals reduce the nitrite to NO). The author also proposes a new hypothesis, the ONS hypothesis, which seeks to explain the simple, yet complex interactions between reactive nitrogen species (RNS), reactive oxygen species (ROS), and reactive sulphur species (RSS) in plants. Because plants are seen by the author as "open systems" having closer contact with the nitrogen, oxygen, and sulphur free radicals existing in the atmospheric environment than do animals, the author proposes a more integrative research process when investigating the NO signaling pathways in plants, including comprehensive studies beginning with molecular interactions to those interactions between organisms. Identifying the underlying mechanisms relating the three reactive series would provide a foundation to understanding the basic "balance science for three distinct dynamic elements (ROS, RNS, RSS)." ROS are generated in the chloroplasts as a toxic byproduct of photosynthesis and CO_2 assimilation; RNS are generated as a byproduct of nitrate assimilation; RSS are a proposed byproduct of sulphur assimilation making all three mutually interactive and causing complexities in the study of free radical biology. Previous thinking and study concluded that NO is a free radical and a toxic environmental pollutant. However further and more recent study has suggested NO as an "essential molecule endogenously produced in the cells…act(ing) as a plant hormone equivalent to ethylene; that is, as a gaseous signal transmitter." The proposed ONS hypothesis should aid in the further exploration of the complexities of NO signaling pathways and the accompanying organismal-environmental interactions.

Vita

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